Immunology 2000 **100** 364–369

Comparative analysis of integrin expression on monocyte-derived macrophages and monocyte-derived dendritic cells

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SUMMARY

Both macrophages (MAC) and dendritic cells (DC) are members of the mononuclear phagocyte system (MPS) with monocytes (MO) as common precursor cells. Cells of the MPS are able to take up, process and present antigens to T lymphocytes, thereby inducing a primary or secondary immune response. Adhesion molecules are of crucial importance for the interaction of antigen-presenting cells with immune cells, especially T lymphocytes. By representational difference analysis, we identified CD49c (VLA-3), a member of the β_1-integrin family of adhesion receptors, as differentiation-associated antigen in MO-derived MAC. In contrast, MO-derived DC did not express CD49c mRNA. These data prompted us to compare the integrin expression pattern of MAC and DC. Both cell types showed a low expression of the α-chains of the β_1-integrins CD49a, CD49b, CD49d and CD49e, whereas a marked difference was observed for CD49c and CD49f. Expression of both integrins increased during MO to MAC differentiation, but was not detectable on DC. In parallel the β_1-chain (CD29) was clearly up-regulated during MO to MAC differentiation but was only weakly expressed on DC. On the other hand, the β_2-integrins CD11a, CD11b, CD11c and CD18 were all expressed on MAC and DC. Beside their role in cell–cell interaction and adhesion, β_2-integrins are also known as possible binding molecules for bacteria and lipopolysaccharide (LPS), especially for high LPS concentrations. Therefore we investigated the LPS response of MAC versus DC in terms of tumour necrosis factor-α (TNF-α) release. DC were less responsive to low doses of LPS, which can easily be explained by the very low CD14 expression on DC compared for MAC. In contrast, the TNF-α response was comparable to MAC when DC were stimulated with high LPS concentrations. Our results show a specific, differentiation-dependent pattern of β_1- and β_2-integrin expression on *in vitro*-generated MAC and DC. We suggest that the high expression of CD11/CD18 on DC could be involved in the LPS binding of DC. As LPS is not only an activation but also a differentiation stimulus for DC, the expression of CD11/CD18 on DC may be important for the successful maturation of DC and thereby the initiation of a primary immune response.

INTRODUCTION

Human blood monocytes (MO) originate from haematopoietic precursors in the bone marrow. They give rise to different types of mature macrophages (MAC), which are distributed ubiquitously in all tissues. *In vitro* culture of peripheral blood MO with human serum[1,2] is an established model for the study of this differentiation process. MAC are important effector cells of the innate immunity and are involved in, for example, phagocytosis and tumour cytotoxicity.

Received 7 December 1999; revised 21 Febraury 2000; accepted 29 February 2000.

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Besides the MO to MAC differentiation pathway, MO can also differentiate into dendritic cells (DC) when cultured in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4).[3,4] *In vitro*-generated MO-derived DC have the ability to take up, process and present foreign antigens. *In vivo* immature DC take up and process antigen in the periphery and then migrate through afferent lymphatic vessels into draining lymph nodes where they present the encountered antigen to T lymphocytes (reviewed in ref. 5). For both MAC and DC adhesion and migration and for the interaction with T lymphocytes adhesion molecules play an important role.

Adhesion molecules can be divided into four families: immunglobulin-like adhesion molecules, selectins, cadherins and integrins.[6]

Integrins are cell surface receptors which participate in

numerous cell–cell and cell–substrate interactions.[7,8] Each integrin is a heterodimer that contains an α- and a β-subunit.[9,10] The integrin receptor family of vertebrates includes at least 16 distinct α-subunits and eight or more β-subunits which can associate to form more than 20 heterodimers. Integrins do not only function as mediators for cell–cell interaction and adhesion, but they also elicit signal transduction events in phagocytes (reviewed in ref. 11). Studies with Chinese hamster ovary (CHO) fibroblasts showed that transfection of CD11c/CD18 rendered the cells responsive to lipopolysaccharide (LPS) and Gram-negative bacteria, independent of CD14 and serum.[12] Other authors suggested that CD11b/CD18 may be involved in signal transduction of glycophosphatyl-inositol (GPI)-anchored surface proteins like CD14 or CD16.[13,14] In the mean time toll-like receptor 2 and 4 (TLR2/4) are known as LPS signal transducer molecules but it is still unknown whether β_2-integrins play a role for LPS binding in situations where CD14 is not present or is limited.

In our initial study we were interested in genes associated with MO to MAC differentiation. We identified the α_3-integrin (VLA-3, CD49c) subunit as MAC-specific cDNA. Further analysis revealed that only MAC showed a strong β_1-integrin expression whereas β_2-integrin expression was comparable on DC and MAC. In addition we analysed the LPS response of both cell types and found that DC stimulated with high LPS concentrations, despite their low CD14 expression, showed a LPS response comparable to MAC. Our findings implicate a possible role for CD11/CD18 as LPS-binding receptors on DC.

MATERIALS AND METHODS

Monocyte isolation and cultivation

Peripheral blood mononuclear cells (MNC) were isolated from leukapheresis concentrates of healthy donors by density gradient centrifugation over Ficoll–Hypaque (Pharmacia, Freiburg, Germany). MO were separated from MNC by counter-current elutriation in a J6M-E Beckman centrifuge (Beckman, Munich, Germany) with a large chamber and a JE-5 rotor at 1100 ***g*** at a flow rate of 110 ml/min in Hanks' balanced salt solution as described previously.[15] Elutriated MO were more than 90% pure as determined by morphology and by antigen expression ($CD14^+$, $CD3^-$, $CD4^-$, $CD8^-$, $CD20^-$) measured by flow cytometry. To induce the *in vitro* differentiation of MO to MAC, purified MO were cultured on hydrophobic Teflon foils for 7–8 days at a cell density of 10^6 cells/ml in RPMI-1640 (Biochrom, Berlin, Germany) supplemented with mercaptoethanol, polyvitamins, antibiotics, pyruvate, non-essential amino acids (all from Gibco BRL, Eggenstein, Germany) and with 2% human pooled AB-group serum as described previously.[1]

For the generation of monocyte-derived DC, MO were cultured for 7–8 days in supplemented RPMI-1640, containing 500 U/ml IL-4 (Promocell, Heidelberg, Germany), 40 ng/ml GM-CSF (Essex, Munich, Germany) and 10% fetal calf serum (FCS; Gibco BRL).

MAC/DC activation

After 7–8 days of culture, monocyte-derived MAC and DC

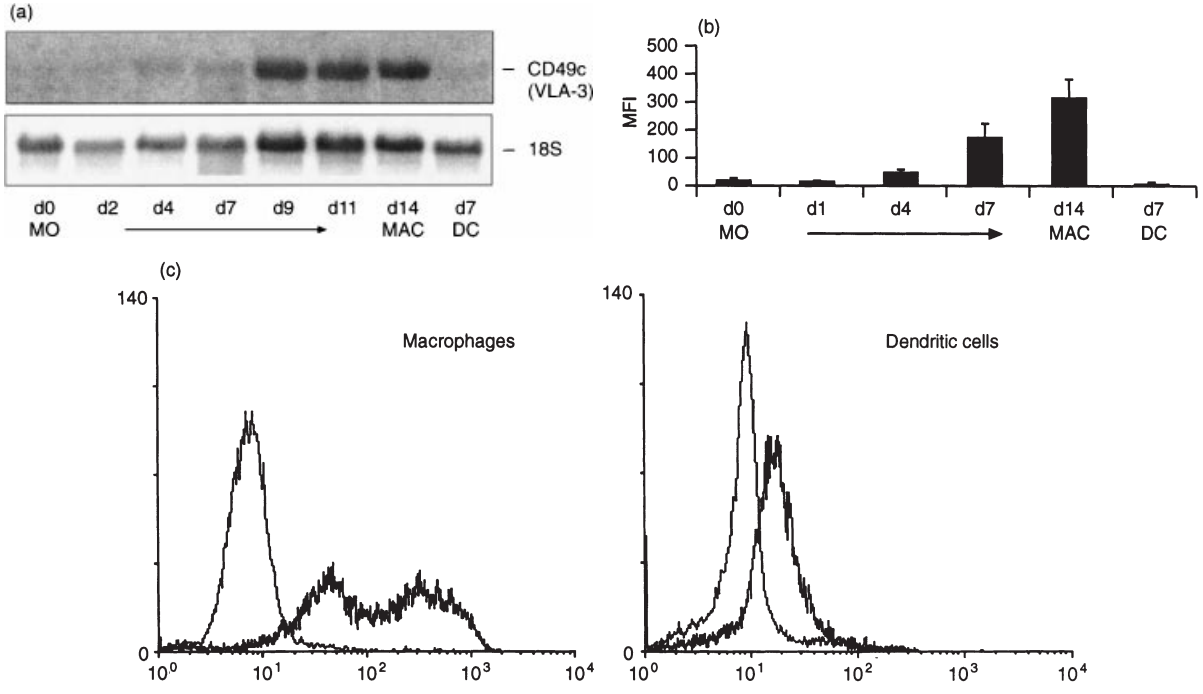

Figure 1. Expression of CD49c (VLA-3) mRNA during MO to MAC differentiation and in DC. (a) MO/MAC were harvested at the indicated time-points (day 0 – day 14), DC at day 7, and total RNA was prepared; 10 μg/lane were loaded and analysed for CD49c mRNA expression by Northern blot analysis. As an internal control the membrane was reprobed with an 18S rRNA oligonucleotide. One representative experiment out of three is shown. (b) Cells (1×10^5) were subjected to FACS analysis at the indicated time-points as described in the Materials and Methods. The mean fluorescence intensity (MFI) ± SEM out of at least three experiments is shown. The mean fluorescence intensity of the isotype control antibody was subtracted. (c) One representative flow cytometry experiment shows the surface expression of CD49c on macrophages and dendritic cells versus an isotype control antibody (IgG).

Table 1. Comparative integrin expression on MO, MAC and DC

Antigen	MO*	MAC†	DC‡
IgG (Isotype control)	8±4	11±5	11±2
MAX.11/CPM	6±3§	370±144	–
CD14	224±33	316±53	10±6
CD49a (α1; VLA-1)	35±18	23±14	11±8
CD49b (α2; VLA-2)	18±17	4±5	2±2
CD49c (α3; VLA-3)	14±8	165±51	7±6
CD49d (α4; VLA-4)	19±5	29±8	13±4
CD49e (α5; VLA-5)	70±28	135±35	152±12
CD49f (α6; VLA-6)	6±5	58±24	4±3
CD29 (β1-chain)	33±7	235±68	155±59
CD11a (αL; LFA-1)	180±46	379±65	113±20
CD11b (αM; CR-3)	155±32	393±122	865±176
CD11c (αX; CR-4)	53±18	612±148	519±76
CD18 (β2-chain)	167±46	426±179	699±52
CD50 (ICAM-3)	74±13	67±27	122±42
CD54 (ICAM-1)	13±3	184±43	218±73
CD102 (ICAM-2)	41±12	12±9	6±4

*MO were purified as described in the Materials and Methods and analysed by flow cytometry.

†MO were grown in 2% human serum in Teflon bags for 7 days to generate *in vitro* differentiated MAC.

‡MO medium was supplemented with 10% FCS, IL-4 and GM-CSF for the differentiation to DC in plastic culture flasks. Non-adherent cells were collected at day 7.

§The values given are the mean fluorescence intensities ± SEM out of at least three FACS experiments with cells of different healthy donors. The mean fluorescence intensity of the isotype control antibody was subtracted.

were harvested, washed with RPMI without serum and cultured for another 24 hr in six-well plates with different concentrations of LPS (*Salmonella abortus equi*, kindly provided by Prof. C. Galanos, Max-Planck Institute, Freiburg, Germany) either in the presence or absence of serum.

Detection of tumour necrosis factor-α (TNF-α)

Supernatants of MAC or DC were analysed for TNF-α by a commercially available enzyme-linked immunosorbent assay (ELISA; R & D Systems, Wiesbaden, Germany).

Representational difference analysis

Total RNA from freshly isolated MO, MO cultivated for 1 day and *in vitro*-differentiated MAC was prepared as described below. Representational difference analysis was performed with pooled MO mRNA from day 0 and day 1 against MAC mRNA as described previously.[16,17] Differently expressed, MAC-specific cDNAs were cloned into pZErO-2 (Stratagene, La Jolla, CA) and sequenced. The DNA sequences obtained were compared with the EMBL, GenBank, PIR, and SwissProt databases by the FASTA[18] computer program (HUSAR computer facilities, Heidelberg, Germany).

RNA preparation and Northern blot analysis

Total RNA was isolated at different time-points from freshly isolated MO, adherent MO, *in vitro*-differentiated MAC, or *in vitro*-differentiated MO-DC by the guanidine thiocyanate/acid phenol method.[19] For Northern blot analysis, different RNA samples (10 μg/lane) were separated by electrophoresis on 1% agarose/formaldehyde gels, transferred to nylon membranes (Magna NT, MSI, Westbrough, MA) and ultraviolet (UV) cross-linked. Hybridization was performed using ^{32}P-labelled cDNA fragments (Random Primed DNA Labelling Kit, Boehringer Mannheim, Mannheim, Germany). To provide an internal control, membranes were reprobed with an oligonucleotide against 18S rRNA labelled by T4-kinase (5′-end labelling kit, Amersham, UK). Autoradiography was performed at −70°.

Flow cytometry analysis

Indirect immunfluorescence staining was performed by incubating 5×10^5 human MO, MAC, or DC with the following monoclonal mouse antibodies for 30 min at 4°: CD11a (clone 25.3.1), CD11b (BEAR1), CD11c (BU15), CD18 (7E4), CD49a (HP2B6), CD49c (C3), CD49e (SAM1), CD49f (GoH3), CD1a (BL6) (all from Immunotech, Hamburg, Germany); CD14 (My4) mouse immunoglobulin G2a (IgG2a; 7T4-1F5) (both from Coulter Electronics, Krefeld, Germany); CD29 (TDM29), CD49b (AK7), CD49d (44H6) (all from Cymbus Biotechnology, Chanders Ford, UK); CD86 (2331; from Pharmingen, San Diego, CA); MAX.11 (own laboratory). Mouse IgG was used as an isotype control. After two washing steps cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (GAM-FITC; from Dianova, Hamburg, Germany) for 30 min at 4°. Two additional washing steps were followed by fixation of MAC/DC with 1% paraformaldehyde in phospate-buffered saline (PBS). Analysis was performed with a fluorescence-activated cell sorter (FACScan) flow cytometer (Becton Dickinson, Mountain View, CA). MAC/DC were gated according to their forward- and side-scattering and the expression of CD14 or CD1a, respectively. The same instrumental setting (FL1426) was used for both cell types.

RESULTS

Expression of CD49c (VLA-3) mRNA and protein in human MO, MAC and DC

Using the representational difference analysis method we analysed mRNA from freshly isolated and overnight-cultured MO (day 0 and day 1) versus *in vitro*-differentiated MO-derived MAC (day 7). We identified a member of the integrin family, CD49c (VLA-3), as differentiation-associated molecule in MAC. This result was confirmed by Northern blot analysis (Fig. 1a) and RT-PCR (data not shown). Accordingly, flow cytometry revealed that CD49c protein was only detectable on *in vitro*-differentiated MAC but not on freshly isolated MO (Fig. 1b). We then investigated whether DC, which can also be

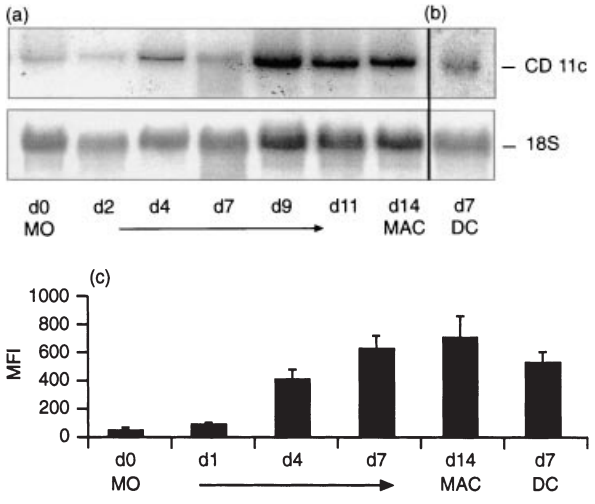

Figure 2. Expression of CD11c mRNA during MO to MAC differentiation and in DC. (a) Northern blot analysis was carried out as described in Figure 1; (b) for the CD11c mRNA expression in DC a longer exposure time of the film was needed to see the specific band clearly. For MO/MAC and DC one representative experiment out of three is shown. (c) FACS analysis was performed as described in the Materials and Methods. The mean fluorescence intensity (MFI) ± SEM out of at least three experiments is shown. The mean fluorescence intensity of the isotype control antibody was subtracted.

generated from MO *in vitro,* would express CD49c. In contrast to MO-derived MAC, CD49c mRNA was not expressed in MO-derived DC (Fig. 1a) and CD49c protein was also not detectable (Fig. 1b). One representative experiment showing CD49c surface expression on macrophages and dendritic cells is shown in Fig. 1(c). These results prompted us to compare the expression pattern of other integrins on MO, MAC and DC by flow cytometry.

Comparative analysis of β_1- and β_2-integrin expression on MO, MAC and DC

Analysing the expression of the different α-chains (CD49a–f) of the β_1-integrins we found a low protein expression of CD49a, CD49b, Cd49d and CD49e on both MAC and DC. Data of the flow cytometry analysis are summarized in Table 1. Similar to CD49c (Fig. 1b), CD49f was up-regulated during MO to MAC differentiation (Table 1), but with a lower mean fluorescence intensity. In contrast, CD49c and CD49f were not detectable on MO-derived DC (Table 1). The common β_1-chain (CD29) was found to be up-regulated along both MO differentiation pathways, although a weaker expression was found on DC (Table 1).

Analysis of the β_2-integrins revealed that CD11a, CD11b, CD11c and CD18 were expressed on both MAC and DC (Table 1 and Fig. 2c). CD11a and CD18 were already present on freshly isolated MO, but CD11b and CD11c expression increased significantly during differentiation of MO into MAC or DC, respectively. The time–course of CD11c mRNA and protein expression during MO differentiation to MAC and DC is shown in Fig. 2.

CD11b and CD11c have been described as receptors for high concentrations of LPS in the absence of CD14.[12, 12] As

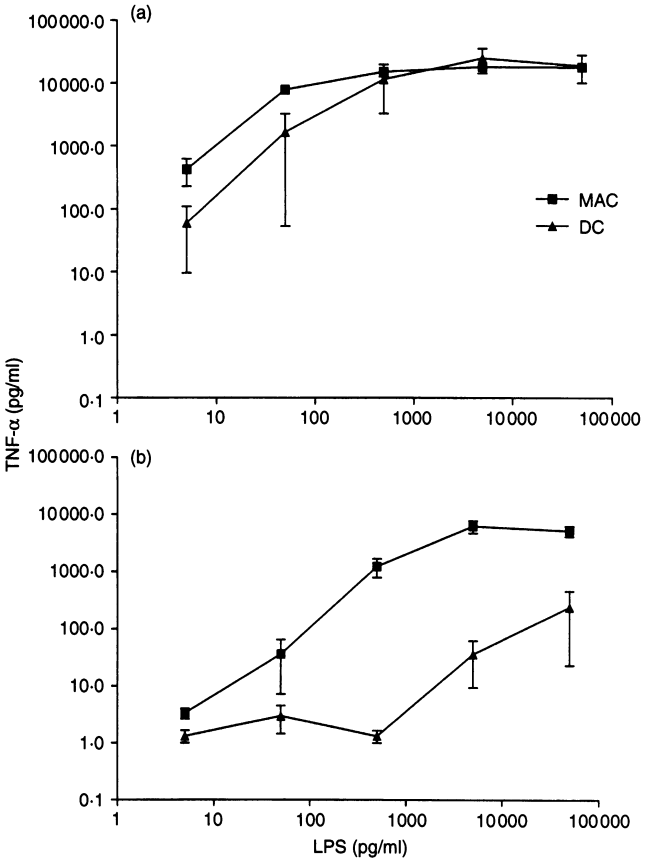

Figure 3. TNF-α production by MAC and DC after LPS stimulation. (a) MAC and DC were obtained by the culture conditions described in the Materials and Methods and harvested on day 7. After two washing steps cells were stimulated with the indicated amounts of LPS for 24 hr under serum conditions. The supernatant was analysed for the presence of TNF-α by cytokine ELISA. Data shown are the mean ± SEM out of four independent experiments. (b) Cells were obtained as described in (a) but stimulated for 24 hr under serum-free conditions. The supernatant was analysed for the presence of TNF-α by cytokine ELISA. Values given are the mean ± SEM for the same four donor cell populations as in Fig. 3(a).

MO-derived DC, in contrast to MAC, expressed almost no CD14 (Table 1) but high amounts of CD11b and CD11c, we compared the LPS response of MAC and DC. Cells were stimulated with LPS and the production of TNF-α was analysed by ELISA.

TNF-α production of MAC and DC after stimulation with LPS

MAC and DC were stimulated overnight with graded doses of LPS in the presence or absence of serum. As expected, MAC produced high levels of TNF-α after LPS stimulation and serum increased the sensitivity for low doses of LPS (Fig. 3). DC were less sensitive to LPS stimulation even in the presence of serum at low LPS doses, however, at high LPS concentrations the TNF-α response was comparable to MAC despite the fact that DC expressed almost no detectable CD14 (Fig. 3).

DISCUSSION

The aim of our initial study was to identify differentiation-associated molecules of *in vitro*-generated MAC. Therefore we used representational difference analysis with MAC versus MO mRNA. With this approach we were able to identify a number of MAC-specific molecules, e.g. human cartilage glycoprotein-39 (HC gp-39),[20,21] and cellular retinoic acid-binding protein II (CRABP II;[22]). Furthermore, we identified CD49c (VLA-3) as a molecule associated with MO to MAC differentiation. Northern blot analysis confirmed the representational difference analysis data and revealed that CD49c is not expressed in MO-derived DC. Thus, CD49c can serve as a marker to distinguish MAC from MO or immature DC. Accordingly, Prieto *et al*. described the expression of CD49c in human MAC.[23]

Performing a more detailed analysis of β_1- and β_2-integrin expression on MO, MAC and DC we found that CD49c and CD49f were up-regulated during MO to MAC differentiation and not expressed on DC. The other α-chains of the β_1-integrins were not regulated during MO maturation, neither to MAC nor DC. CD49c and CD49f are both members of the β_1-integrin family and function as receptors for extracellular matrix components like collagen, laminin, or fibronectin.[22,25] *In vivo* these integrins might be important for MAC adherence to sites of injury or inflammation. As matrix components have been shown to modulate MAC function[26] we suggest that CD49c and CD49f play an important role for the activation of MAC but not DC.

Studies on the expression of the β_2-integrin family showed that all of these antigens were expressed already on MO, but were up-regulated during MO differentiation into MAC or DC. Beside their role as adhesion molecules, CD11b and CD11c were discussed as LPS receptors in CHO transfection studies.[12] As DC expressed high levels of CD11b and CD11c but almost no CD14, we examined the LPS response of DC in comparison to MAC. MAC and DC showed a LPS response comparable to MAC at high LPS concentrations. However, the LPS response of DC was much weaker at low concentrations and DC failed to respond to low LPS concentrations under serum-free conditions. Verhasselt and colleagues[27] suggested that the LPS response of DC is mainly mediated via soluble CD14 (sCD14), a protein present in normal serum. This is in accordance with our findings as we found no LPS answer at low LPS concentrations under serum-free conditions where no sCD14 is present. However, stimulation with high LPS concentrations cannot be blocked by anti-CD14 antibodies and therefore seems to be at least partially independent of CD14 action.[28] Up to now, only indirect evidence has existed that CD11b/c may be relevant for LPS-binding in DC. In neutrophils it was demonstrated recently by Troelstra *et al*. that CD11b is important for LPS-binding but not LPS activation.[29] In addition, the signal transducer for LPS, toll-like receptor-4 (TLR-4), may also be responsible for the CD14 independent LPS response of DC, as in transfection studies with TLR4, a minimal NF-κB activation could also be triggered without CD14 co-transfection.[30] Further experiments will have to clarify the role of β_2-integrins for DC activation.

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